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Complement levels and leucocyte phagocytosis in newborn babies

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Summary
Newborn babies face higher risk of infection than adults, but the immunological basis of this observation is yet to be fully explained particularly in babies of different gestations and birth weights. Sixty-two (62) adults, 55 full-term babies, 18 low birth weight babies and 44 normal birth weight babies were considered for the study. B-lymphocytes and T-lymphocytes were enumerated by EAC-rosette and E-rosette respectively. Leucocyte migration and intracellular killing were assessed by percentage migration index (% M.I), percentage Candidacidal index (% C.I) and bacterial stimulated nitroblue tetrazolium (NBT) dye reduction index (%NBT) respectively. Also, serum levels of C3 and C5 were measured by single radial immuno-diffusion method. Percentage T cell, C3, C5, %NBT and %C.I were lowest in low birth weight babies but % B cell was lowest in full term babies while normal birth babies had least %M.I. The present study suggests that gestational age and birth weight affect different aspects of immune response.

Keywords: *Birth weight, gestational ages, phagocytosis, leucocyte migration, neonates.*

Résumé
Les nouveaux-nés sont dans le plus grand risque d'infection que les adultes. Mais la base immunologique de cette observation n'ai pas encore bien connue particulierement chez les bébés en diffrent stage de gestations et leur poids. Soixante-deux (62) adultes, 55 bébés a terme, 18 bébés ayant un poids corporel baisse et 44 bébé s ayant un poids normal etaient consideres. B-lymphocytes et T-lymphocytes etaient enumeres par EAC-rostte et E-rosette respectivement. La migration des leucocytes et la mort intracellualre etaient evaluees par pourcentage d'index de migration(%M.I) ; pourcentage d'index candidacidal(%C.I) et bacterio-tetrazolum mitroblue stumilee (NBT) et l'index de reduction du dye(%NBT) respectivement. En addition, les niveaux du serum et C3 et C5 etaient mesures par la methode de simple diffusion radiale. Le pourcemtage de T cells, C3, C5 n% NBT et % C.I etaient tres faible chez les bebes ayant un poids faible a la naissance mais le % de B cells était plus faible chez les bébés a terme, alorsque les bébés normaux avaient moins de %M.I. Cette étude suggére que l'age de gestation et le poids a la naissance affecte different aspects de l'immunité normale.

Introduction

Several studies have shown that neonates are more susceptible to infections when compared with adults. Among suggested reasons are the: reduced expression of neutrophil adhesion molecules [1], abnormal chemotaxis, bone marrow exhaustion, complement deficiency [2,3], incompetence of T cells at birth [4], immaturity of antigen presenting cells and co-stimulatory molecule expression in the neonates [5,6]. However, the extent to which deficiency of cell mediated or humoral mediated immunity explains hypo-responsiveness in newborn babies of different gestations and birth weights is poorly understood.

In the study reported here, the cellular and humoral components of phagocytosis in neonates were compared with those of the adults. The outcome of this study is expected to improve therapeutic approaches to neonatal septicemia and also provide valuable information on the mechanism involved in normal inflammatory response.

Materials and methods

Blood samples:
Informed consents were obtained from the mothers of the recruited babies and the adults before the collection of blood samples. The babies considered for this study were delivered at Adeoyo State Hospital and University College Hospital, Ibadan, Nigeria while the adults were members of staff from the hospitals. Blood was collected from the umbilical cords of 177 infants that were separated based on gestational ages and birth weight at delivery. Fifty-five (55) were full term babies ($\oplus$37 weeks gestational age), 18 were low birth weight babies (< 2.5 kg birth weight). They were all born by normal delivery. From each baby, 8-12 ml of cord blood was collected in a tube containing anticoagulant (heparin). Ten ml blood samples were also collected from apparently healthy adults by venepuncture into a tube containing heparin.

Preparation of blood lymphocytes from whole blood:
Blood lymphocytes were isolated from whole blood by the method described by Ling and McLennan [7]. Between three to five ml of heparinised blood was each delivered into a universal bottle and 5 ml of medium (Hanks solution - heparin solution) was mixed with the blood. This was carefully layered over 4 ml of lymphoprep (Pharmacia, USA), centrifuged at 850 x G for 20 minutes and the mononuclear cells between the plasma and lymphoprep was carefully removed into a clean plastic centrifuge tube. The cells were washed 3 times with Hanks-heparin solution and the lymphocyte number was counted with haemocytometer. The % cell viability was evaluated in 0.5% Trypan blue dye.

Enumeration of T-lymphocytes:
This was based on the method described by Salimonu *et al* [8]. 0.25 ml of 4 x 10^6 lymphocytes/ml Hanks solution was mixed with 0.25 ml of 0.5% suspension of washed sheep red blood cells (SRBC) in PBS, pH 7.2. Adsorbed heat inactivated foetal calf serum (FCS, 0.1 ml) was added, incubated at 37°C for 5 minutes, centrifuged for 5 minutes at 100 x G and re-incubated at 4°C overnight. The % rosetting cell was determined by counting 200 lymphocytes in haemocytometer. A rosette forming lymphocyte was defined as one that bound 3 or more (SRBC) [8]. The assay was performed in duplicates.

Enumeration of B-lymphocytes:
The procedure for the enumeration of B-lymphocytes was as described above for the enumeration of T-lymphocytes except

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that the SRBC used were sensitised with sub-agglutinating dose of guinea-pig complement by gentle mixing. 0.25 ml of 5% presensitised SRBC was mixed with 0.25 ml of 4 x 10^6 lymphocytes/ml, incubated at 37°C for 45 minutes, this was centrifuged at 200 x G for 3 minutes and the percentage rosetting lymphocytes counted using haemocytometer.

Leucocyte migration inhibitory factor assay
The method previously described was followed [9]. Two ml of heparinised blood was mixed with equal volume of 3% dextran solution and this was allowed to stand at 37°C for 1 hr. The leucocyte rich supernatant was spun at 300 x G for 10 minutes, washed in 15% FCS and the number adjusted to 1 x 10^8 cells/L before being filled into capillary tubes. These were spun at 850 x G for 10 minutes, cut at packed cell-medium interface, placed in a migration chamber and filled with either medium (15% FCS) or antigen-medium solution. The antigen-medium used was 1:20 dilution of BCG (Bacille Calmette Guerin) in 15% FCS and incubated at 37°C in CO_2 for 18 hrs. The migration area was traced unto transparent paper and measured by counting the number of small squares enclosed on a graph paper. The percentage migration index (% M.I) due to the antigen was calculated thus: %M.I=T/C.x 100 where T is the area of migration in antigen solution and C is the area of migration in medium.

Collection of neutrophils:
Neutrophil suspension was prepared from fresh three to five ml of unclotted blood as described by Strober[10]. Five ml of blood was mixed with equal volume of 6% Hanks solution and allowed to stand at 20°C for 45 minutes. The leucocyte rich plasma was harvested, centrifuged at 850 x G for 10 minutes at 5°C, resuspended in 5ml of 0.9% NaCl and was gently layered on 5ml lymphoprep solution. This was spun at 800 x G for 40 minutes at 20°C, the saline and lymphoprep layers were aspirated leaving neutrophil / red blood cell pellets. The RBCs were lysed with 10ml of cold 0.2% NaCl for 30 seconds, after which 10ml cold 1.6% NaCl was added to restore the isotonicity. Neutrophil pellet that was obtained by spinning at 850 x G for 6 minutes at 5°C was resuspended in cold Hanks solution. The total number of the neutrophil was recorded and the number adjusted to 5 x 10^6 cells / ml.

Percentage neutrophil Candidacidal index (%C.I):
Percentage C.I was determined using the abilities of neutrophils to kill *Candida albicans* as previously described [11]. A suspension of 24 hr culture of *C. albicans* was made in Hanks solution. This was adjusted to 5 x 10^6 cells/ml of Hanks solution and viability of the neutrophils was confirmed to be 95% by the Trypan-blue dye. To a mixture of 0.25ml autologous plasma obtained from spun blood, 0.25 ml Hanks solution, 0.25 ml of *Candida* suspensions and 0.25 ml of 5 x 10^6 neutrophil suspension were added. A similar set up was made for the control tube except that neutrophil suspension was omitted. The tubes containing the mixture were incubated for 1 hr with shaking at every 15 minutes. At the end of this period, 0.25 ml of 2.5% sodium desoxycholate was added to each mixture to lyse neutrophils but not the *Candida*. One (1) ml of 0.01% methylene blue was added to stain dead *Candida*. Methylene blue was carefully removed with a pipette after pelleting the *Candida* by spinning. The organism was resuspend in a drop of Hanks solution. Two hundred (200) *Candida* was counted using haemocytometer and the percentages of dead *Candida* (stained cells) was determined from this.

Nitroblue Tetrazolium dye reduction index (%NBT):
This was based on the method of Feigin *et al* [23]. For the stimulated NBT procedure, 50µl of NBT solution, 25µl heparinized blood and 25µl of stimulant solution (*E.coli* lipopolysaccharide) were mixed gently, incubated at 37°C for 10 minutes and for further 10 minutes at room temperature. Thick smear of the mixture was prepared, air dried for 10 minutes and treated with undiluted Wright stain for 15 seconds and diluted Wright stain (1:1) for 30 seconds before rinsing in water and air-dried. 100 neutrophils were counted under oil immersion objective and neutrophils showing dark formazan deposit were recorded as positive. Percentage bacterial stimulated NBT index was calculated as neutrophils with dark formazan deposit (positive) divided by neutrophil count.

Determination of C3 and C5 levels:
Single radial immunodiffusion method using rabbit anti-human C3 and C5 anti-sera (Sigma, USA) were used to measure C3 and C5 levels respectively [22]. The diameter of precipitin rings formed after antigen-antibody reaction in a buffered agar gel is proportional to the concentration of C3 and C5 present in the plasma. A volume of an optimally diluted monospecific antiserum was mixed with noble agar and poured on glass plate. Wells of equal diameter were cut in the antibody/agar mixture. The wells were filled with test or standard serum. The plates were incubated for 18 hours at room temperature. After incubation, the diameters of precipitin rings were measured using a Hyland viewer with measured using a Hyland viewer with a micrometer eyepiece.

Statistical analysis:
Statistical analysis was performed by calculating the mean, standard deviation, ANOVA and Students *t* test. *P*-value less than 0.05 was significant.

Results

The result presented in Table 1 shows that % B cells were higher in all the groups of babies compared with the adults. Normal birth weight babies had the highest % B cell. Contrarily, the % T was significantly lower in the babies compared with adults. Babies with low birth weight had lowest % T cell (Table 1). Percentage M.I were higher in all groups of babies compared with

Table 1: Comparison of T-lymphocyte and B-lymphocyte levels (x ± 1S.D) in newborn babies and adults

	Full term (n=55)	Low birth weight (n=28)	Normal birth weight (n=44)	Adults (n=62)	F-, p-values
T cells (%)	48±6*	30±3*(	39±6*	61±10	71.2, 0.000
B cells (%)	26±7*	34±3*	36±5*	20±4	81.3; 0.000

* *Significantly different from adults (P<0.05)*
(*Significantly different from normal birth weight babies (P<0.05)*

adults. Low birth weight babies had the highest % M.I. In Table 2, % C.I was lowest in low-birth weight babies compared with normal weight babies. Though % NBT dye reduction index was similar in both full-term babies and the adults, other groups of the babies had significantly lower NBT dye reduction index compared with the adults. The levels of C3 and C5 were significantly

reduced in the babies compared with the adults. Moreover, C3 and C5 were lower in low birth weight- babies compared with normal weight- babies respectively.

Table 2: Comparison of percentage migration index, percentage candidacidal index, NBT dye reduction index and complement levels (x ± 1S.D) of babies compared with adults

	Full term (n=55)	Low birth weight (n=28)	Normal birth weight (n=44)	Adults (n=62)	F-, p-values
% M.I	45±24*	61±15*(	38±11	35±15	11.81; 0.000
% C I	41±23*	38±9*(	42±13*	50±13	4.31; 0.009
% NBT	60±15	42±9*(	58±11*	64±12	10.59; 0.000
C3	35±9*	33±9*	34±12*	70±13	9.61, 0.000
C5	4±0.9*	3±0.8*	3.9±1.1*	6±1	6.03, 0.000

* *Significantly different from mothers (P<0.05).*
(*Significantly different from normal birth weight babies(P<0.05) C3 and C5 were measured in mg/dl*

Discussion

The result shows that out of a total 117 babies considered for this study, 55(47%) full term babies, 18(15%) were low birth weight babies and 44(38%) normal birth weight babies. The percentage prevalence of pre-term babies or low birth weight babies was less compared with the values obtained in the same environment years ago [22]. This may be a reflection of improved pre-natal care.

This study demonstrates deficiencies of phagocytosis in human neonates. Normally, prenatal period is a time of little antigenic challenge, reduced immunoglobulin synthesis and few differentiated plasma cells [17], therefore lower number of B-lymphocytes in neonates is expected. Our finding of higher proportion of B-lymphocytes in neonates compared with the adults may represent a shift of B-lymphocytes from primary lymphoid organ to the blood circulation in the neonates. Evidence of depressed number of T-lymphocytes in neonates compared with adults is consistent with the reported small size and immaturity of thymus and spleen of newborn babies [18].

Stages of polymorphs bactericidal activity involve migration, attachment, engulfing and intracellular destruction that were assessed as percentage miogration index, percentage candidacidal index and NBT dye index respectively. Babies with low birth weight had highest percentage migration index compared with other groups of babies. This may be explained by reduced number of T-lymphocytes, defective T lymphocytes response to antigenic stimulation, reduced leucocyte migration inhibition factor (LMIF) secretion or defective sensitivity of leucocytes to secreted LMIF. This supports a previous observation that neonates are prone to *Listeria* as a result of decreased generation of interleukins, which attracts macrophage to the site of infection [19]. The proportion of T lymphocytes was higher in adults compared with all groups of babies, thus explaining reduced % M.I in the adults compared with the babies. The extent of leucocyte migration is determined by LMIF concentration, thus the higher LMIF concentration, the lower the % M.I. Ward and Becker [24] have shown that two cell-bound serine esterases are required for cell migration. One of these esterases exists in, or on, the leucocytes in an activated state. The other is enzymatically inert and becomes activated following interaction with the complement system. Qualitative or quantitative deficiencies of one, or both, of these esterases might explain the deficient migratory response by the neonatal leucocytes.

Nitroblue tetrazolium (NBT) dye reduction index by polymorphonuclear leucocytes from cord blood samples of babies (particularly the low birth weight babies) was lower than that of adults. This may be as a result of either reduced production of O_2 radical or depressed production of leucocyte pyrogen in neonates. Stimulated neutrophils undergo O_2 dependent microbial activity to generate H_2O_2 that reduces NBT to produce formazan deposits. Therefore reduced O_2 consumption and depressed generation of reactive O_2 species may be responsible for the observed NBT dye reduction especially in low birth weight babies.

It is possible that the cellular defects in phagocytic activities found in the neonatal leucocytes may be related to developmental immaturity of immune system. Humoral enhancement of phagocytosis is dependent, at least in part, on the serum complement system. Chemotactic and phagocytic activities have been associated with C3 and C5 as well as trimolecular complex of C5, C6, C7 [25]. In the present study, quantitative deficiencies of C3 and C5 have been demonstrated in all neonates. Miller [25] showed that addition of C3 and C5 to sera of neonates significantly increased phagocytic and to a lesser extent chemotactic activity. While other serum factors may be involved, it appears that a deficiency of neonatal sera in generation of C3 and C5 results in defective phagocytosis.

The present data on levels of C3 and C5 in babies may suggest a gradual increase of complement components with increasing age. Whether this increase is due to fetal production or increasing trans-placental passage remains to be established. Adinolfi *et al* [26] suggested that fetal production play a role. Certainly, the reduction in complement components of low birth weight babies may explain increased susceptibility to infections as a result of defective opsonisation.

From this study, it could be concluded that phagocytes in neonates are not fully immunocompetent at birth and that neonates are likely to be deficient in inflammatory responses.

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